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Identification of the Parental Species of a Putative Hybrid Spruce *Picea* × *notha* Using DNA Markers with Contrasting Modes of Inheritance

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Picea × *notha*, described by Rehder in 1939, is thought to be a putative hybrid between pollen receptive *P. glehnii* and pollen donating *P. jezoensis* var. *hondoensis*; however, such hybrid is questionable because the distributions of *P. glehnii* and *P. jezoensis* var. *hondoensis* do not overlap naturally. Recently, a natural hybrid between *P. glehnii* and *P. jezoensis* var. *jezoensis*, which is morphologically similar to *P.* × *notha*, was genetically determined. Therefore, the goal of this study was to identify the parental species of *P.* × *notha* using maternally inherited mitochondrial (mt), paternally inherited chloroplast (cp), and biparentally inherited nuclear (n) DNA markers and to elucidate the similarity of *P.* × *notha* and natural hybrids occurring in Hokkaido. Genetic analyses indicated that *P.* × *notha* harbored *P. glehnii* mtDNA, *P. jezoensis* var. *jezoensis* or *P. jezoensis* var. *hondoensis* cpDNA, and *P. glehnii* and *P. jezoensis* var. *jezoensis* nDNA almost equally. This clearly indicated that *P.* × *notha* is an F_1 hybrid between pollen receptive *P. glehnii* and pollen donating *P. jezoensis* var. *jezoensis* and that it is similar to natural hybrids found in Hokkaido. This is the first report to demonstrate the parental species of *P.* × *notha* and its natural distribution in Hokkaido, Japan.

Key words: chloroplast DNA, distribution, mitochondrial DNA, nuclear microsatellite, *Picea glehnii*, *Picea jezoensis* var. *hondoensis*, *Picea jezoensis* var. *jezoensis*

Species of *Picea* (Pinaceae) are among the most important components of the boreal and temperate forest biomes (Farjon 1990). Species of *Picea* are also known to hybridize naturally (Wright 1955, Perron & Bousquet 1997, Hamilton *et al*. 2013, Haselhorst & Buerkle 2013, Luckwood *et al*. 2013, Sun *et al*. 2014, Aizawa *et al*. 2016, Tsuda *et al*. 2016).

Picea × *notha* Rehder is a questionable putative hybrid described by Rehder in 1939 (Figs. 1 & 2). According to his description, the hybrid was discovered among approximately 15 planted trees raised as *P. glehnii* (F. Schmidt) Mast. (Sakhalin spruce) in the Arnold Arboretum of Harvard University, from seeds received from the Governmental Forestry School, Tokyo, Japan in 1894. Rehder (1939) assumed that the tree was a hybrid between pollen receptive *P. glehnii* and pollen donating *P. jezoensis* (Siebold et Zucc.) Carrière var. *hondoensis* (Mayr) Rehder (Hondo spruce) because the tree had intermediate morphology between the two species: it had pilose branchlets like *P. glehnii* but flat needle leaves like *P. jezoensis* var. *hondoensis*. However, the assumption by Rehder about the parental species of the *P.* × *notha* is questionable because the ranges of *P. glehnii* and *P. jezoensis* var. *hondoensis* do not overlap in nature; *P. glehnii* mainly occurs in Hokkaido whereas *P. jezoensis* var. *hondoensis* occurs in central Honshu, Japan (Fig.

FIG. 1. Holotype of *Picea* × *notha* deposited in A (Arnold Arboretum of Harvard University). The digital image of the holotype was obtained from the Digital Collection of the Harvard University Herbaria.

FIG. 2. Young (second-year) branchlet with somewhat thin pubescence on the sample used in this study, which was collected from a living tree of *Picea* × *notha* (Accession No. 13406) from the Arnold Arboretum of Harvard University (A).

3). Thus, the parental species of *Picea* × *notha* remain uncertain.

In Pinaceae, mitochondrial (mt) DNA is maternally inherited, chloroplast (cp) DNA is paternally inherited (Neale & Sederoff 1989, Wagner 1992) and nuclear DNA is inherited biparentally. Thus, analysis using DNA markers with contrasting modes of inheritance allows for the identification of both parental species of hybrids in Pinaceae (Watano *et al*. 1996, Isoda *et al*. 2000, Watano *et al*. 2004). In Hokkaido, Japan, a natural hybrid between *Picea jezoensis* var. *jezoensis* (Yezo spruce) and *P. glehnii* is known to occur (Hamaya *et al*. 1989). Recently, Aizawa *et al*. (2016) verified the natural hybrid using nuclear microsatellite, cpDNA, and mtDNA markers and demonstrated that pubescence on young branchlets is an effective morphological marker for identifying the natural hybrid. Because the *P.* × *notha* is reported to have pubescent branchlets, it could be similar to the natural hybrid from Hokkaido.

The goal of this study, therefore, was to identify the parental species of *Picea* × *notha* using DNA markers with contrasting modes of inheritance and to elucidate the similarity between *P.* × *notha* and the natural hybrid between *P. jezoensis* var. *jezoensis* and *P. glehnii* on Hokkaido.

Materials and Methods

Sampling, observation of pubescence on young shoots, and DNA extraction for Picea × notha

We obtained a silica-dried shoot collected from the type tree of *Picea* × *notha* (accession No. 13406; Figs. 1 & 2) from the Arnold Arboretum of Harvard University (A). *Picea* × *notha* exhibited somewhat thin pubescence (Fig. 2) on young branchlets, determined based on the five grades proposed by Hamaya *et al*. (1989). Total DNA was extracted from 50 mg of silica-dried needles using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

DNA samples used for analyses

For genotyping of cpDNA and mtDNA, we used the 12 samples of *Picea jezoensis* var. *hondoensis*: three samples from each of the four populations (Oze, Mt. Norikura, Mt. Fuji, and Mt. Odaigahara, Japan; Fig. 3) that were used in Aizawa *et al*. (2007). For nuclear microsatellite analyses, we used 52 samples including *P.* × *notha* and 51 DNA samples from previous studies (Aizawa *et al*. 2007, 2009, 2015, 2016): three individuals from each of five natural populations (Horoshika Pass, Akan, Ochiishi, Oshamanbe, and Hayachine; Fig. 3) across Hokkaido and northern Honshu for *P. glehnii*, five natural populations (Soya, Shiretoko, Furano, Ochiishi, and Mt. Shiribeshi; Fig. 3) across Hokkaido for *P. jezoensis* var. *jezoensis*, five natural populations (Oze, Mt. Kusatsushirane, Mt. Norikura, Mt. Fuji, and Mt. Odaigahara; Fig. 3) across Honshu for *P. jezoensis* var. *hondoensis*, and two artificial F_1 hybrids between *P. glehnii* and *P. jezoensis* var. *jezoensis* [*g* (V-2) × *j* (mixed pollen) and *g* (V-103) × *j* (mixed pollen); Aizawa *et al*. (2016)] and their pollen receptive and candidate pollen donating parents.

Design of the diagnostic organelle DNA markers

Previously developed diagnostic cpDNA and mtDNA markers (Aizawa *et al.* 2016) were used to distinguish between the *Picea jezoensis* var.

jezoensis and *Picea glehnii* (Table 1). We used as the cpDNA marker the species-specific single nucleotide polymorphism (SNP), i.e., guanine (*G*-type cpDNA) in *P. glehnii* and thymine (*J*-type cpDNA) in *P. jezoensis* var. *jezoensis* at position 1,454 in the sequences of the *trnC* (GCA)-*trnD* (GUC) intergenic regions (hereafter *trnC-trnD*) of both species (accession Nos. DQ010561 and DQ010567; Ran *et al*. 2006; Table 1). The *trnC-trnD* was amplified using a primer pair of Demesure *et al*. (1995). We used as the mtDNA marker the section A in domain IV of the second intron (called intron b/c) of the mitochondrial gene that codes for NADH dehydrogenase subunit 1, which is variable and produces mtDNA haplotype H4 (*G*-type mtDNA) for *P. glehnii* and H3 (*J*-type mtDNA) for *P. jezoensis* var. *jezoensis* (Table 1). The *nad1* intron b/c was amplified using a primer pair of Demesure *et al.* (1995). For cpDNA, we performed one-pass direct sequencing of a single strand to genotype the species-specific bases using an internal primer (*petN3G*; Ran *et al*. 2006) for *trnC-trnD*. Similarly, for mtDNA, we performed one-pass direct sequencing of a single strand of section A in domain IV of *nad1* intron b/c using an internal primer (*nad1Rint2*; Aizawa *et al*. 2016). For *P.* × *notha*, the entire sequence of the PCR product for *nad1* intron b/c in mtDNA was determined using an additional internal primer used in Aizawa *et al*. (2015). The protocols for the PCR and sequencing have been described elsewhere (Aizawa *et al*. 2007, 2015).

Nuclear microsatellites

This study required diagnostic nuclear DNA markers capable of differentiating among *Picea glehnii*, *P. jezoensis* var. *jezoensis*, and *P. jezoensis* var. *hondoensis* to identify the parental species of *P.* × *notha*. Previous studies differentiated *P. glehnii* and *P. jezoensis* var. *jezoensis* (Aizawa *et al*. 2015), and *P. jezoensis* var. *jezoensis* and *P. jezoensis* var. *hondoensis* (Aizawa *et al*. 2009), using four nuclear microsatellite loci; therefore, we selected these four loci (Table 2). In addition, we selected five loci (Table 2) based on an initial screening with nuclear microsatellite loci newly developed for *P. jezoensis* var. *jezoensis* (Iwaizumi *et al*. 2015); the five loci exhibited polymorphism at each locus for the three spruces and had easily discernible alleles for scoring. PCR reactions were performed in 10-μL volumes. For the four loci from Aizawa *et al*. (2015), the reaction mixture contained approximately 20 ng genomic DNA, 0.1 mM of each dNTP, 1× PCR buffer, 2 mM $MgCl_2$, 0.5 U *Taq* polymerase (Promega, Madison, WI, USA), and 0.2 μM of each primer. For the five loci from Iwaizumi *et al*. (2015), the mixture contained approximately 20 ng genomic DNA, 0.2 mM of each dNTP, 1× PCR buffer, 2 mM $MgCl_2$, 0.5 U *Taq* polymerase (Promega), and 0.15 μM of each primer. The PCR thermal profile was as follows: an initial denaturing step for 5 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at the annealing temperature (Table 2), and 45 s at 72°C, and a final elongation step at 72°C for 10 min, in a GeneAmp 2720 PCR System (Applied Biosystems, PE Corp., Foster City, CA, USA). The forward sequence of each primer pair was labeled with a fluorescent dye (6-FAM, VIC, NED, or PET). The genotypes were determined using an ABI 3500 Genetic Analyzer and GENEMAPPER v.4.1 (Applied Biosystems).

Data analysis

Identification of the parental species of *Picea* × *notha* was accomplished using nine nuclear microsatellite loci and a model-based Bayesian clustering algorithm implemented in STRUCTURE v.2.3.4 (Pritchard *et al*. 2000). The STRUCTURE algorithm estimates allele frequencies for each gene pool and population memberships for every individual (Hubisz *et al.* 2009). We used the LOCPRIOR model, which considers taxon information (*P. glehnii*, *P. jezoensis* var. *jezoensis*, and *P. jezoensis* var. *hondoensis* and the hybrid) as priors (Hubisz *et al*. 2009), an admixture model, and the correlated allele frequencies model (Falush *et al*. 2003). We used hybrid information as a prior for *P.* × *notha* because the results of cpDNA and mtDNA analyses indicated that it was a possible F1 hybrid (see Results). Before the STRUCTURE analysis, we tested genotypic disequilibrium using FSTAT 2.9.3 (Goudet 2001) for all pairs of loci using 720 permutations. STRUC-

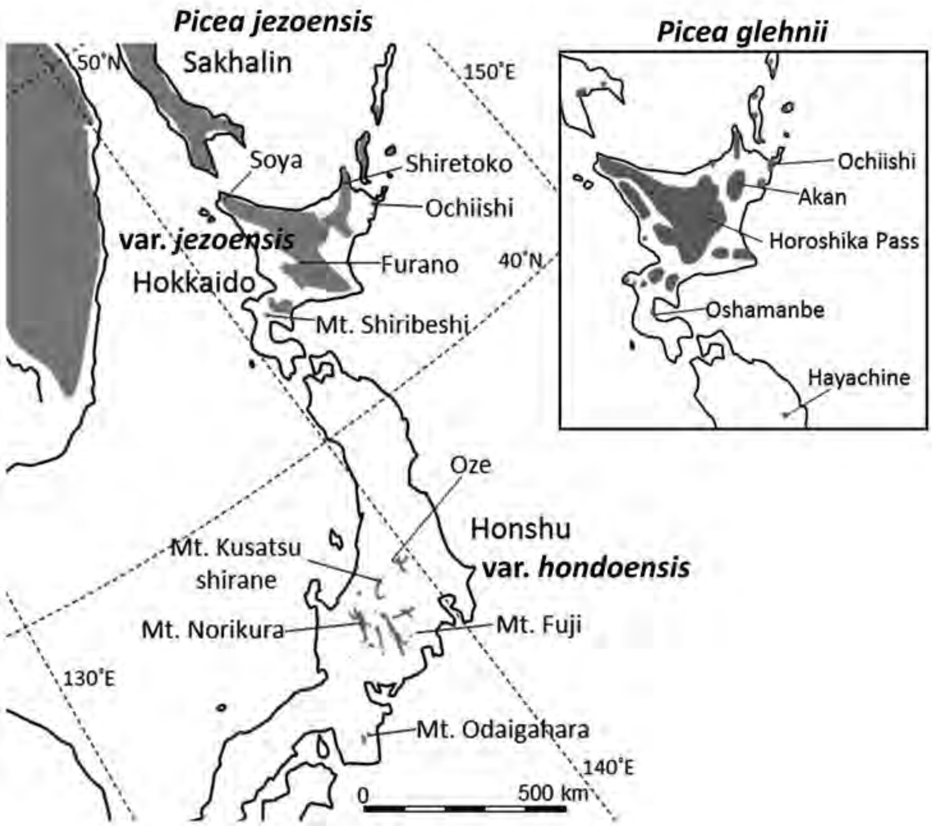

FIG. 3. Natural distributions of *Picea jezoensis* var. *jezoenses*, *P. jezoensis* var. *hondoensis*, and *P. glehnii* in Japan and adjacent regions (areas in gray) and populations used for analyses.

TABLE 1. Summary of the genetic analysis using DNA markers with contrasting modes of inheritance.

taxon	mtDNA†	cpDNA	nDNA
Picea glehnii	*G* (H4)‡	*G*	*G*
Picea jezoensis var. *jezoensis*	*J* (H3)‡	*J*	*Y*
Picea jezoensis var. *hondoensis*	*H* (H6)	*J*	*H*
Picea × *notha*	*G* (H4)	*J*	*G*/*Y*

†Nomenclature of haplotypes in parentheses was defined by Aizawa *et al.*(2015); ‡H4 and H3 are species-specific for *Picea glehnii* and *P. jezoensis* var. *jezoensis*, respectively; however, H2 has been observed at low frequencies in both taxa (Aizawa *et al.* 2015, 2016).

TURE was run for 10,000 Markov chain Monte Carlo (MCMC) iterations after a burn-in period of 20,000. STRUCTURE was run 20 times independently at K = 1–5. The results of STRUCTURE were harvested using STRUCTURE HARVESTER (Earl and vonHoldt 2012). The outputs of 20 independent runs were integrated using CLUMPP v.1.1.2 (Jakobsson & Rosenberg 2007) and visualized using DISTRUCT v.1.1 (Rosenberg 2004).

Results

The results of cpDNA and mtDNA genotyping for 12 samples of *Picea jezoensis* var. *hondoensis* and those for *P. glehnii* and *P. jezoensis* var. *jezoensis* (Aizawa *et al*. 2015, 2016) are summarized in Table 1. The results indicated that all *P. jezoensis* var. *hondoensis* harbored *J*-type cpDNA as does *P. jezoensis* var. *jezoensis*, indicating that *P. jezoensis* var. *jezoensis* and *P. jezoensis* var. *hondoensis* could not be distinguished using this cpDNA marker (Table 1). In mtDNA, all

TABLE 2. Characteristics of the nuclear microsatellite markers used for 52 samples, including *Picea* × *notha*, *P. glehnii*, *P. jezoensis* var. *jezoensis*, and *P. jezoensis* var. *hondoensis*, and artificial F_1 hybrids between *P. glehnii* and *P. jezoensis* var. *jezoensis*, in this study.

Locus	T_A	Size range	N	N_A	H_S	H_T
UAPgAC/AT6	66	94-119	52	6	0.665	0.774
SpAGG3	60	102-138	52	18	0.759	0.850
SpAGD1	60	112-160	52	19	0.944	0.929
SpAGC1	60	73-126	52	23	0.822	0.899
bcpj0123	60	132-186	52	23	0.899	0.939
bcpj 0147	60	143-186	52	18	0.665	0.917
bcpj0666	60	69-109	52	12	0.759	0.819
bcpj0073	60	116-185	52	25	0.944	0.943
bcpj0960	60	122-166	52	24	0.822	0.907

T_A, annealing temperature (°C); Size range, PCR product size range (base pair); N, number of samples analyzed; N_A, number of alleles detected; H_S,gene diversity within taxa (*Picea glehnii*, *P. jezoensis* var. *jezoensis*, and *P. jezoensis* var. *hondoensis* and hybrids); H_T, overall gene diversity.

Picea jezoensis var. *hondoensis* harbored the H6 haplotype that was previously observed in *P. jezoensis* var. *hondoensis* (Aizawa *et al*. 2015). *Picea* × *notha* harbored the *J*-type cpDNA and *G*-type mtDNA (H4 haplotype), indicating that the type tree of *P.* × *notha* is an F_1 hybrid between a pollen receptive *P. glehnii* and pollen donating *P. jezoensis* var. *jezoensis* or *P. jezoensis* var. *hondoensis*. The sequences obtained have been deposited in GenBank under Accession Nos. LC223599–LC223605.

Results of the STRUCTURE analysis using nine nuclear microsatellite loci for 52 samples, containing the *Picea glehnii*, *P. jezoensis* var. *jezoensis*, *P. jezoensis* var. *hondoensis*, artificial F_1 hybrids, and *P.* × *notha*, indicated that the highest log probability of the data and highest value of ΔK (Evanno *et al*. 2005) were both found at $K = 2$ (data not shown). However, because our goal in this study was to examine the parental species of *P.* × *notha* and the results were almost consistent at $K = 3$–5, we fixed K at 3. The results at $K = 3$ indicated that the nuclear gene pools in P. *glehnii*, *P. jezoensis* var. *jezoensis*, and *P. jezoensis* var. *hondoensis* were explicitly distinct from each other (Fig.4). The results also clearly showed that the artificial F_1 hybrids and *P.* × *notha* had mixed ancestry: nearly equal contribution of *P. glehnii* and *P. jezoensis* var. *jezoensis* (Fig. 4; Table 1).

Discussion

Parental species of Picea × notha

It is inscrutable why Rehder (1939) reported *Picea jezoensis* var. *hondoensis*, instead of *P. jezoensis* var. *jezoensis* to be the paternal parent of *P.* × *notha*. The genetic analyses using DNA markers with contrasting modes of inheritance indicated that *P.* × *notha* harbored *G*-type mtDNA, *J*-type cpDNA, and showed nuclear admixture between *P. glehnii* and *P. jezoensis* var. *jezoensis*, which unambiguously demonstrated that *P.* × *notha* is an F_1 hybrid between a pollen receptive *P. glehnii* and pollen donating *P. jezoensis* var. *jezoensis*. Aizawa *et al*. (2016) revealed natural hybrids between *P. glehnii* and P. *jezoensis* var. *jezoensis* in central Hokkaido. Therefore, these natural hybrids in central Hokkaido are *P.* × *notha*. The natural distribution of *P.* × *notha* has remained unknown (Farjon 1990); it was not listed by Hayashi (1960), Yamazaki (1995), and Yonekura (2012). This is the first study, to our knowledge, that describes the natural distribution of *Picea* × *notha* in Japan.

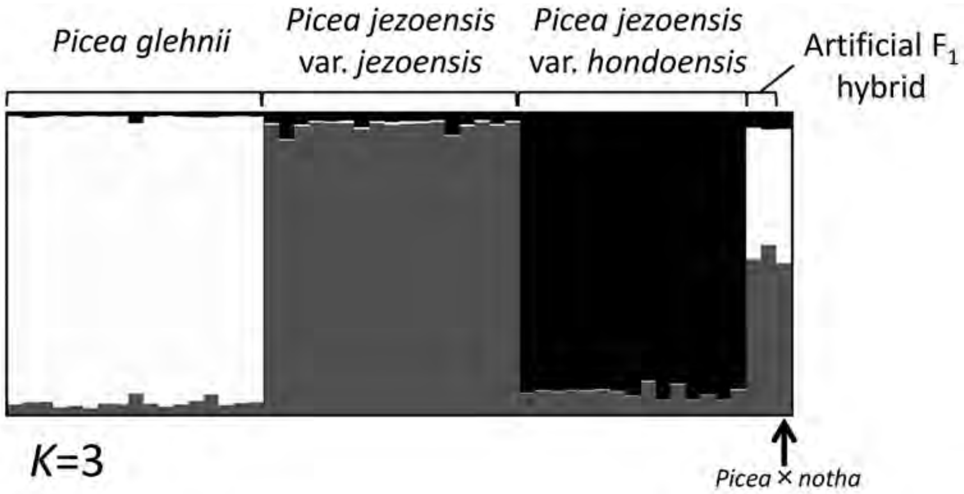

FIG. 4. Result of the Bayesian clustering STRUCTURE defined using nine nuclear microsatellite loci for *Picea glehnii*, *P. jezoensis* var. *jezoensis*, and *P. jezoensis* var. *hondoensis*, artificial F_1 hybrids, and *P.* × *notha* at $K = 3$. Each individual is represented by a thin vertical line.

Picea × notha Rehder, J. Arnold Arbor. 20: 85–86 (1939). *Picea glehnii* (♀) × *Picea jezoensis* var. *jezoensis* (♂).

This hybrid differs from *Picea jezoensis* var. *jezoensis* and var. *hondoensis* in having pubescence on young, especially current year branchlets and broader less undulate cone scales; it differs from *P. glehnii* in having less pilose branchlets, more compressed (flat) needle leaves, and cones with flexible, narrower, and distinctly erose-denticulate scales.

Typus. USA, MA, Suffolk County, Boston, Arnold Arboretum, Jamaica Plan. (*A. Rehder & E. J. Palmer 13406*, *28 Sept*, *1936*, holo- in A, digital image!)

Japanese name. Ke-ezomatsu, nov.

Distribution. Hokkaido (Yamabe, Furano-shi). Our herbarium investigation found a specimen from Tôberi, Tokachi. In addition, Hayama *et al*. (1989) reported hybrids from Oketo-cho, Tokoro-gun; Yukomanbetsu, Higashikawa-cho, Kamikawa-gun; and Biei-cho, Kamikawa-gun.

Habitat. Natural forests with mixed *Picea glehnii* and *P. jezoensis* var. *jezoensis*.

Notes. Aizawa *et al*. (2016) reported the natural hybrid was also formed by crosses of *Picea jezoensis* var. *jezoensis* (♀) × *P. glehnii* (♂). Some specimens from outside Hokkaido, namely, from middle and northern Sakhalin, Kamchatka, maritime Russia, and northeast China, deposited in TI, SAP, and SAPS, have pubescence on young branchlets (Aizawa unpubl. data). In most of the regions, except for northeast China and maritime Russia, where Korean spruce (*P. koraiensis* Nakai) co-occurs with *P. jezoensis* var. *jezoensis*, a sympatric congener species is unknown. Therefore, those specimens are unlikely to be hybrids. Further genetic study is necessary to confirm this.

The origin of the seeds of the type specimen of *Picea* × *notha* in A is unresolved. Rehder (1939) stated that the seeds were received in 1894 from the Governmental Forestry School, Tokyo. However, Tokyo Forestry School (Tokyo Sanrin Gakko) had already been abolished in 1886, moved to Komaba, Tokyo, and become the College of Agriculture of the Imperial University. Tokyo Forestry School was located on the premises of the Tree Experimental Station of the Forestry Bureau (Sanrinkyoku Jumokushikenjo) at Nishigahara, Tokyo, until the school was moved. The Tree Experimental Station had central roles in collecting seeds of trees from across Japan and exchanging seeds of trees with foreign countries during the Meiji Era. The Tree Experimental Station also had an experimental forest for testing growth, plantation, and cultivation of tree seedlings on its premises. We therefore examined

documents from the Tree Experimental Station, including a position diagram and list of planted trees, published in 1879 (Geography Bureau 1879). The document indicated *Picea glehnii*, *P. jezoensis* var. *jezoensis*, and *P. jezoensis* var. *hondoensis* were not planted at that time. According to a document involved in the collection and transportation of seeds from 1878 to 1881 in Ando (1966), the Tree Experimental Station collected seeds of trees in natural forests in Japan through their branch offices to distribute to prefectures in Japan and to foreign countries. The seeds of *P. glehnii*, including *P.* × *notha*, could also have been collected from natural forest(s) on Hokkaido.

Other specimens examined. JAPAN, Hokkaido, Furano, The University of Tokyo Hokkaido Forest: Forest compartment #7a, approximately 620-m altitude, g×jA No.1 (tree ID corresponds to Hamaya *et al.* 1989), 43°18′42.61″N, 142°35′42.90″E, Dec. 15, 2006, *M. Aizawa et al. 06121501* (TOFO); g×jA No.3, 43°18′34.14″N, 142°35′42.63″E, Dec. 15, 2006, *M. Aizawa et al. 06121502* (TOFO); g×jA No.6, 43°18′34.30″N, 142°35′44.94″E, Dec. 15, 2006, *M. Aizawa et al. 06121503* (TOFO); Forest compartment #7b, approximately 500-m altitude, g×jB No.5, 43°17′49.22″N, 142°35′28.40″E, Dec. 15, 2006, *M. Aizawa et al. 06121504* (TOFO); Tôberi hen, Tokachi, Jun. 25, 1884, no collector name (TI).

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